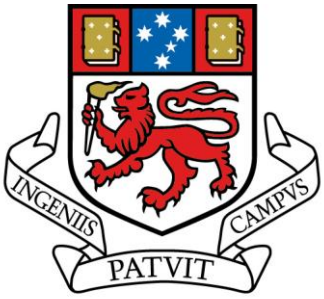

UNIVERSITY OF TASMANIA

ADVANCED GAS CHROMATOGRAPHY WITH MASS SPECTROMETRY FOR PHYTOANALYSIS OF HOP (*Humulus lupulus* L.)

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BSc, MSc

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Natural Sciences

University of Tasmania

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I. Statement of authenticity

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Dandan Yan

Melbourne, June 2018

II. Abstract

The phytochemical composition of hop (*Humulus lupulus* L.) makes it a vital raw material for the brewing industry. However, the separation, detection, and identification of all components present within hops remains a major challenge. This thesis focuses on a range of developments and applications of advanced gas chromatography (GC) techniques, hyphenated with high resolution mass spectrometry (MS) for untargeted and/or targeted profiling of hop secondary metabolites in *H. lupulus* L. essential oils.

One-dimensional gas chromatography (1D GC) is still the mainstay analytical method in plant essential oil analyses. Phytochemical characterisation of essential oils derived from 30 representative Australian hop genotypes, including experimental hybrid genotypes and commercial cultivars, using high resolution GC coupled with quadrupole accurate mass time-of-flight MS (Q-TOFMS) is described. The diversity of volatile phytoconstituents among the analysed samples was critically evaluated and interpreted in terms of their genetic and biogeographical origins, and in light of an understanding of the biosynthetic processes that result in accumulation of flavour-relevant metabolites in mature hop cones. This study highlights the potential and indicates the limitations of one-dimensional GC analysis of complex plant samples.

The potential of comprehensive two-dimensional gas chromatography (GC×GC) combined with Q-TOFMS, to perform the untargeted profiling of essential oils derived from a representative range of hop genotypes, is discussed. Comprehensive overview and distinct differences of metabolic profiles were readily observed (based on 2D or 3D chromatograms) due to the orderly distributions of metabolites in the 2D separation space, allowing simple and fast fingerprinting of different hop genotypes. Distinguishable chemotype patterns were displayed among experimental and commercial hop genotypes, indicating genetic diversity among the samples studied. A complex array of secondary metabolites was detected, and oxygenated sesquiterpenes were proposed as a key chemical identifier group for different genotypes. The markedly different metabolite profiles were further interpreted through chemometric

analysis, which allow the classification of three major chemotypes among analysed genotypes. The findings indicate that this high resolution chromatographic approach has great potential towards improved methodologies for better understanding of hop chemistry.

Next, development of a four-column multiplexed technique with two independent two-dimensional column ensembles (2GC×2GC), employing an additional gas supply and electronic pressure control at the midpoint between the two dimensions, is outlined. Appropriate carrier-gas flow control at the junction of the first-dimension (^{1}D) and second-dimension (^{2}D) columns permits the possibility and simplicity of implementing GC×GC and 2GC×2GC experiments. Comparison of analyses with and without independent ^{2}D flow control, and the importance of applying flow control to adjust the separation speed in ^{2}D were outlined. The analytical performance of the proposed multiplexed approach was demonstrated by the analyses of hop essential oil with two different column combinations. The unique ability to design two entirely independent conventional GC×GC separations for each injection, yielding two independent GC×GC chromatograms viewed in a single window leading to an appreciable gain of productivity was demonstrated.

Lastly, a novel sequential three-dimensional GC hyphenated with Q-TOFMS (3D GC–Q-TOFMS) approach for profiling of secondary metabolites in complex plant extracts is described. The integrated system incorporates a preliminary non-polar first-dimension ($^1D_{np}$) separation step, prior to a microfluidic heart-cutting (H/C) of a targeted region(s) into a polar second-dimension ($^2D_{PEG}$) column for multidimensional separation (GC_{np}–GC_{PEG}). For additional separation, the effluent from $^2D_{PEG}$ can then be modulated according to a GC×GC process, using an ionic liquid phase as a third-dimension ($^3D_{IL}$) column, to produce a sequential GC_{np}–GC_{PEG}×GC_{IL} separation. The described integrated system can be used in a number of modes, but one useful approach is to target specific classes of compounds for improved resolution. The analytical performance and applicability of the proposed approach is demonstrated and discussed through the separation and detection of the oxygenated sesquiterpenes in hop essential oil and agarwood (*Aquilaria malaccensis*) oleoresin. Improved resolution

and peak capacity is illustrated through the progressive comparison of the tentatively identified components for GC_{np}–GC_{PEG} and GC_{np}–GC_{PEG}×GC_{IL} methods. The described methodology should be a valuable adjunct for the improved characterisation of complex plant samples, particularly in the area of plant metabolomics.

III. Publications during enrolment

2018:

- D.D. Yan, Y.F. Wong, R.A. Shellie, P.J. Marriott, S.P. Whittock, A. Koutoulis, Assessment of the phytochemical profiles of novel hop (*Humulus lupulus* L.) cultivars: A potential route to beer crafting, *Food Chemistry* **2019**, *275*, 15–23.

- D.D. Yan, Y.F. Wong, S.P. Whittock, A. Koutoulis, R.A. Shellie, P.J. Marriott, Sequential hybrid three-dimensional gas chromatography with accurate mass spectrometry: A novel tool for high-resolution characterization of multicomponent samples, *Analytical Chemistry* **2018**, *90*, 5264–5271.

- Y.F. Wong, D.D. Yan, R.A. Shellie, D. Sciarrone, P.J. Marriott, Rapid plant volatiles screening using headspace SPME and person-portable gas chromatography–mass spectrometry, *Chromatographia* **2019**, https://doi.org/10.1007/s10337-018-3605-2

2017:

- D.D. Yan, Y.F. Wong, L. Tedone, R.A. Shellie, P.J. Marriott, S.P. Whittock, A. Koutoulis, Chemotyping of new hop (*Humulus lupulus* L.) genotypes using comprehensive two-dimensional gas chromatography with quadrupole accurate mass time-of-flight mass spectrometry, *Journal of Chromatography A* **2018**, *1536*, 110–121.

- D.D. Yan, L. Tedone, A. Koutoulis, S.P. Whittock, R.A. Shellie, Parallel comprehensive two-dimensional gas chromatography, *Journal of Chromatography A* **2017**, *1524*, 202–209.

2016:

- A. Diwan, B. Singh, T. Roychowdhury, D.D. Yan, L. Tedone, P.N. Nesterenko, B. Paull, E.T. Sevy, R.A. Shellie, M. Kaykhaii, M.R. Linford, Porous, high capacity coatings for solid phase microextraction by sputtering, *Analytical Chemistry* **2016**, *88*, 1593–1600.

IV. Conference presentations

"Sequential three-dimensional gas chromatography with accurate mass spectrometry: a novel tool for high-resolution characterization of multicomponent samples" Oral presentation at *15th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology*, Cardiff, United Kingdom, January 2018.

"Evaluation of three-dimensional gas chromatography with accurate mass spectrometry for high resolution characterization of multicomponent samples" Oral presentation at *41st International Symposium on Capillary Chromatography and 14th GC×GC Symposium*, Fort Worth, Texas, United States of America, May 2017.

"Performance evaluation of applying flow control in multiplexed dual dimensions comprehensive two-dimensional gas chromatography" Poster presentation at *ACROSS International Symposium on Advances in Separation Science 2*, Hobart, TAS, Australia, December 2016.

"Multiplexed dual dimensions comprehensive two-dimensional gas chromatography (2GC×2GC) with contra-directional thermal modulation and flow control" Poster presentation at *40th International Symposium on Capillary Chromatography and 13th GC×GC Symposium*, Riva del Garda, Trento, Italy, May 2016.

V. Statement of co-authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

Paper 1: Assessment of the phytochemical profiles of novel hop (*Humulus lupulus* L.) cultivars: A potential route to beer crafting; Located in Chapter 2.

Candidate (70%), Yong Foo Wong (5%), Robert A. Shellie (5%), Philip J. Marriott (5%), Simon P. Whittock (10%), Anthony Koutoulis (5%).

Paper 2: Chemotyping of new hop (*Humulus lupulus* L.) genotypes using comprehensive two-dimensional gas chromatography with quadrupole accurate mass time-of-flight mass spectrometry; Located in Chapter 3.

Candidate (70%), Yong Foo Wong (8%), Laura Tedone (2%), Robert A. Shellie (5%), Philip J. Marriott (5%), Simon P. Whittock (5%), Anthony Koutoulis (5%).

Paper 3: Parallel comprehensive two-dimensional gas chromatography; Located in Chapter 4.

Candidate (75%), Laura Tedone (5%), Anthony Koutoulis (5%), Simon P. Whittock (5%), Robert A. Shellie (10%).

Paper 4: Sequential hybrid three-dimensional gas chromatography with accurate mass spectrometry: A novel tool for high-resolution characterization of multicomponent samples; Located in Chapter 5.

Candidate (70%), Yong Foo Wong (10%), Simon P. Whittock (5%), Anthony Koutoulis (5%), Robert A. Shellie (5%), Philip J. Marriott (5%).

Details of the Authors roles:

Candidate proposed idea, developed and validated methods, analysed and interpreted all data, fully drafted manuscript. Another co-authors assisted with refinement and presentation.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published peer-reviewed manuscripts contributing to this thesis:

Signed:

Prof. Anthony Koutoulis	Prof. Mark Hunt
Main Supervisor	Head of School
School of Natural Sciences	School of Natural Sciences
University of Tasmania	University of Tasmania
Date: June 11, 2018	**June 13, 2018**

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VII. List of Abbreviations

AED	Atomic emission detector
β	Phase ratio
CCD	Counter-current distribution
CT	Cryogenic trapping device
DXN	Desmethylxanthohumol
DTD	Direct thermal desorption
1D GC	One-dimensional gas chromatography
^{1}D	First-dimension
^{2}D	Second-dimension
DS	Deans switch
d_f	Film thickness
3D	Three-dimensional
4D	Four-dimensional
FID	Flame-ionisation detector
FPD	Flame-photometric detector
FFNSC	Flavour and fragrance of natural and synthetic compounds
GC	Gas chromatography
GC-O	GC-olfactometry
GC-MS	Gas chromatography mass spectrometry
GC×GC	Comprehensive two-dimensional gas chromatography
HS-SPME	Headspace solid-phase microextraction
H/C MDGC	Heart-cut multidimensional gas chromatography
IXN	Isoxanthohumol
I.D.	Internal diameter
ISTD	Internal standard
IOFI	International organisation of the flavour industry
K_c	Distribution constant
k	Retention factor
LC	Liquid chromatography

MS	Mass spectrometry
MDC	Multidimensional chromatography
MDCC	Multidimensional comprehensive chromatography
M_R	Modulation ratio
NIST	National institute of standards and technology
n_c	Peak capacity
NPD	Nitrogen-phosphorous detector
NCD	Nitrogen-chemiluminescence detector
6-PN	6-Prenylnaringenin
8-PN	8-Prenylnaringenin
P_M	Modulation period
qMS	Quadrupole mass spectrometry
Q-TOFMS	Quadrupole accurate mass time-of-flight mass spectrometry
RI	Retention index
RRF	Relative-response factors
SCD	Sulfur-chemiluminescence detector
SFC	Supercritical fluid chromatography
TLC	Thin-layer chromatography
t_R'	Adjusted retention time
t_M	Holdup time
t_R	Retention time
TOFMS	Time-of-flight mass spectrometry
μECD	Micro-electron capture detector
w	Peak width at half-height or at base
XN	Xanthohumol

CHAPTER 1

Introduction

1.1 Brief introduction to hop (*Humulus lupulus* L.)

Hop (*Humulus lupulus* L.), a plant indigenous to Europe, Asia, and North America, is an important industrial crop cultivated worldwide. *H. lupulus* L. plants are almost exclusively used as an essential ingredient in beer manufacturing to impart an attractive aroma as well as typical bitterness to the final beverage [1-3]. Besides its brewing value, extracts obtained from hop have been used in phytopharmaceuticals, dietary supplements, functional beverages and cosmetics [4-7]. Hop is a perennial, dioecious climbing plant of the *Cannabaceae* family (**Figure 1.1 A**). Female strobiles (commonly called cones, **Figure 1.1 B**) of hop contain numerous glandular trichomes (lupulin glands), which are mainly located in the outer lower surface of bracteoles and in the whole surface of the perianth (**Figure 1.1 C**) [8,9]. These glands contain a unique and complex pool of secondary metabolites biosynthesised in the foliar mesophyll, including hop resins, polyphenols and essential oils [9,10].

Figure 1.1 (A) Hop in the field; (B) Hop cones; (C) Lupulin glands (yellow) in the hop cone.

1.1.1 Chemical composition of hop

Hop resins

The major hop resins are bitter acids as a source of the bitterness of beer. Their important components are *α*-acids and *β*-acids, whose bittering potential differs markedly. *α*-acids contain three major congeners, namely cohumulone (**1a**), *n*-humulone (**1b**), and adhumulone (**1c**). They are different in the acyl side chain, and their ratio of concentration is dependent upon cultivar and timing of sampling during fruit maturation. During the brewing process, *α*-acids are thermally isomerised into iso-*α*-acids via an acyloin-type ring contraction, leading to the formation of two main epimeric isomers: *cis*-iso-*α*-acids (**3a-c**) and *trans*-iso-*α*-acids (**4a-c**) (**Figure 1.2**) [11]. Iso-*α*-acids are key players in the organoleptic profile of beer, and contribute to the overall bitterness in beer [12,13]. Their impact on bitterness, beer foam stability, and inhibition of Gram-positive bacterial growth are well described [14-16]. Similarly, the *β*-acids are also composed of three major congeners, namely, colupulone (**2a**), *n*-lupulone (**2b**), and adlupulone (**2c**) (**Figure 1.2**). The biological behavior of *β*-acids is entirely different from that of *α*-acids because of differences in their molecular structures. *β*-acids do not undergo isomerisation upon wort boiling. However, they are prone to extensive oxidative degradation, mainly at the double bond of the prenyl side chains. The presence of bitter-tasting transformation products of *β*-acids, *e.g*., hulupones and humulinones, were confirmed in recent studies [17-20], and beers hopped by partially oxidized pure *β*-acids showed pleasant sensorial bitterness [18,21]. Different hop cultivars are typically classified into three groups on the basis of their *α*-acids content: high-*α* bitter hops, intermediate-*α* bitter hop and aroma (noble) hop [22].

Hence, particular variety specific characteristics are important for the classification and use of particular hop cultivars in brewing. Such traits might include the *α*/*β* ratio, percent of *α*- and *β*-acids, cohumulone ratio (defined by the amount of cohumulone per total *α*-acids), and composition of essential oils [23].

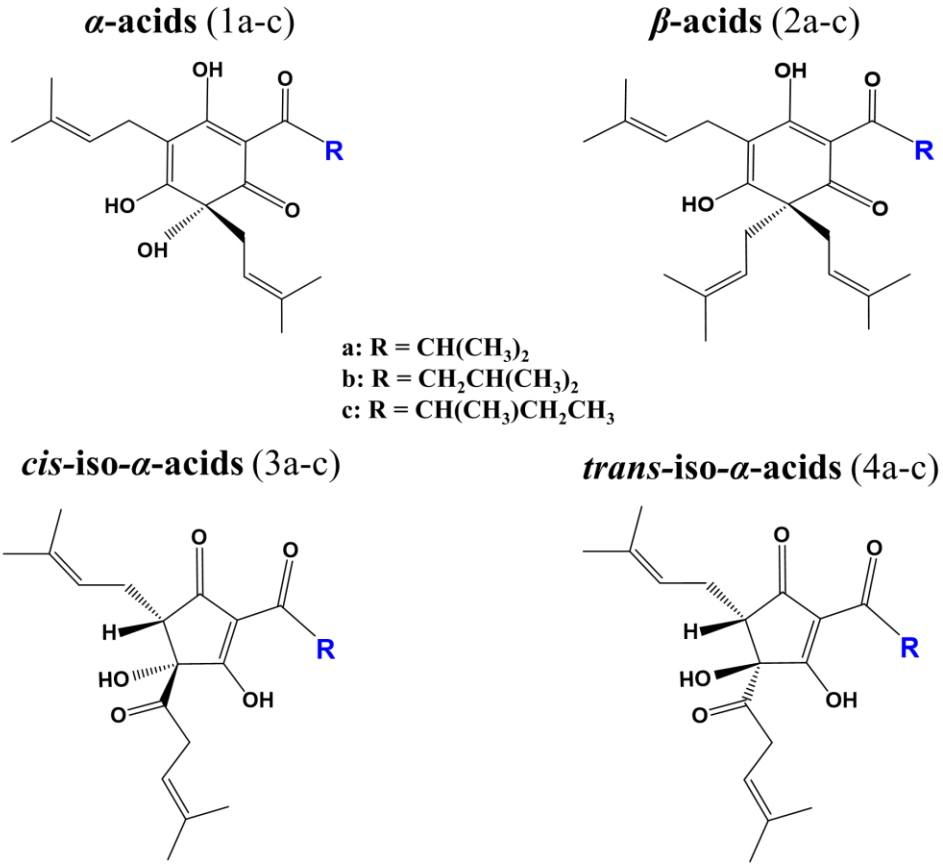

Figure 1.2 Chemical structures of individual classes of the hop bitter acids: *α*-acids, including cohumulone (**1a**), *n*-humulone (**1b**), and adhumulone (**1c**); *β*-acids, including colupulone (**2a**), *n*-lupulone (**2b**), and adlupulone (**2c**); *cis*-iso-*α*-acids, including *cis*-isocohumulone (**3a**), *cis*-iso-*n*-humulone (**3b**), and *cis*-isoadhumulone (**3c**); and *trans*-iso-*α*-acids, including *trans*-isocohumulone (**4a**), *trans*-iso-*n*-humulone (**4b**), and *trans*-isoadhumulone (**4c**). R corresponds to each homolog.

Hop polyphenols

Hop contains a wide range of polyphenolic compounds, which can be further divided into various chemical classes. In general, the most important hop flavonoids are

xanthohumol (XN) and the related prenylflavonoids, such as desmethylxanthohumol (DXN), isoxanthohumol (IXN), 8-prenylnaringenin (8-PN), and 6-prenylnaringenin (6-PN) (**Figure 1.3**) [5,24,25]. In addition to their contributions to brewing, hop prenylflavonoids has been considered as healthy agent because of their anticancer, antioxidant, anti-inflammatory, and antimicrobial properties [5]. Specifically, they have been reported to have the capability of reducing the cholesterol level, protecting against the cardiovascular diseases, and inhibiting many enzymes, *e.g.*, 8-PN is known as the most potent phytoestrogen [5,25]. The composition of polyphenols in hops mainly depends on the hop variety, cultivation zone, harvesting technique, age and storage condition [2,25]. The importance of polyphenols is not only because of their contribution to beer flavour, preventing beer from oxidation and improving flavour stability, but also due to their role in the formation of non-biological haze, which limits the shelf-life of bottled beers [2].

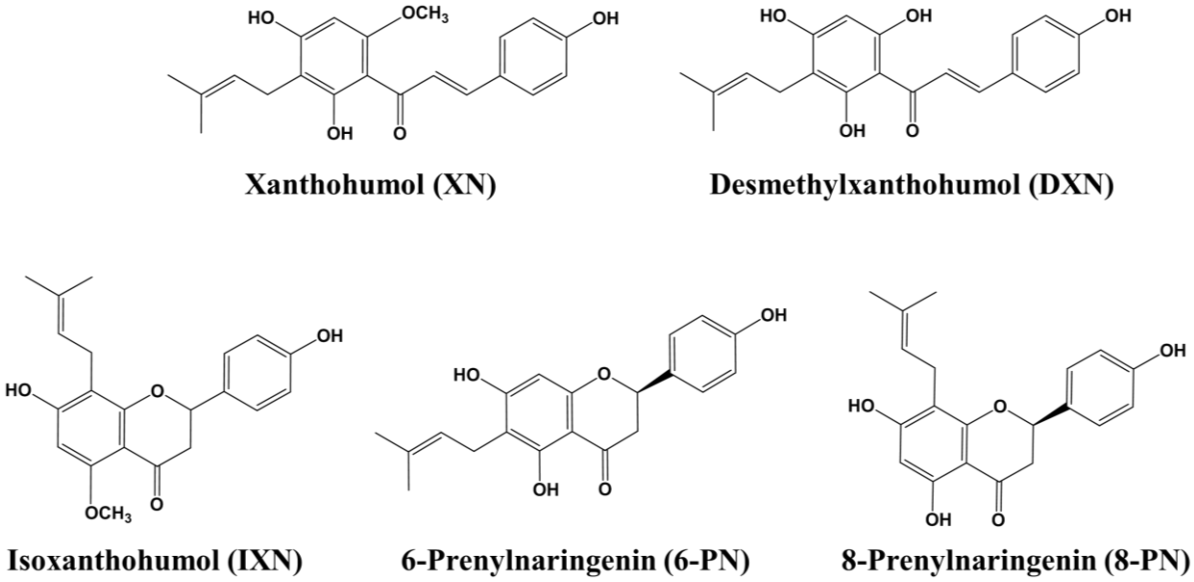

Figure 1.3 Chemical structures of common polyphenols detected in hop and beer samples.

Hop essential oils

Different hop varieties can confer distinct flavour and aroma characteristics to beer, which can, in part, be attributed to the different chemical composition of their essential oils (the volatiles of hops) [26]. Hop essential oil is a very heterogeneous and complex mixture, potentially containing over 1,000 volatile compounds from a wide range of chemical classes [27]. The vast majority of volatiles are either by-products of plant metabolism (biosynthesis) or evolving from secondary reactions (oxidation and hydrolysis) of volatile and nonvolatile precursor molecules. Hop oil constituents are generally classified into three chemical groups, namely hydrocarbon compounds, oxygenated compounds, and sulfur-containing compounds [28], contributing to the unique organoleptic properties of particular cultivars observed in beer (a general overview of the classification is shown in **Figure 1.4**).

The hydrocarbons can be classified into three distinct groups, monoterpenes, sesquiterpenes, and aliphatic hydrocarbons, which account for 50–80% of the total essential oil [28]. The hydrocarbon group is very volatile and can be easily oxidised and/or polymerised. Therefore, most of these compounds are lost or transformed during hop storage and wort boiling process, resulting in only trace amounts are found in beer [29,30]. Myrcene, one of the most potent odorants of hop aroma related to its geranium-like notes [31], is the predominant component of the monoterpene group, typically accounting for > 30% of the total oil content [28]. The sesquiterpenes are highly reactive and give rise to many oxidised terpenes in the oxygenated fraction. The most important and abundant components of the sesquiterpene group are α-humulene and β-caryophyllene, both reported to have a spicy and woody odour [32]. These three

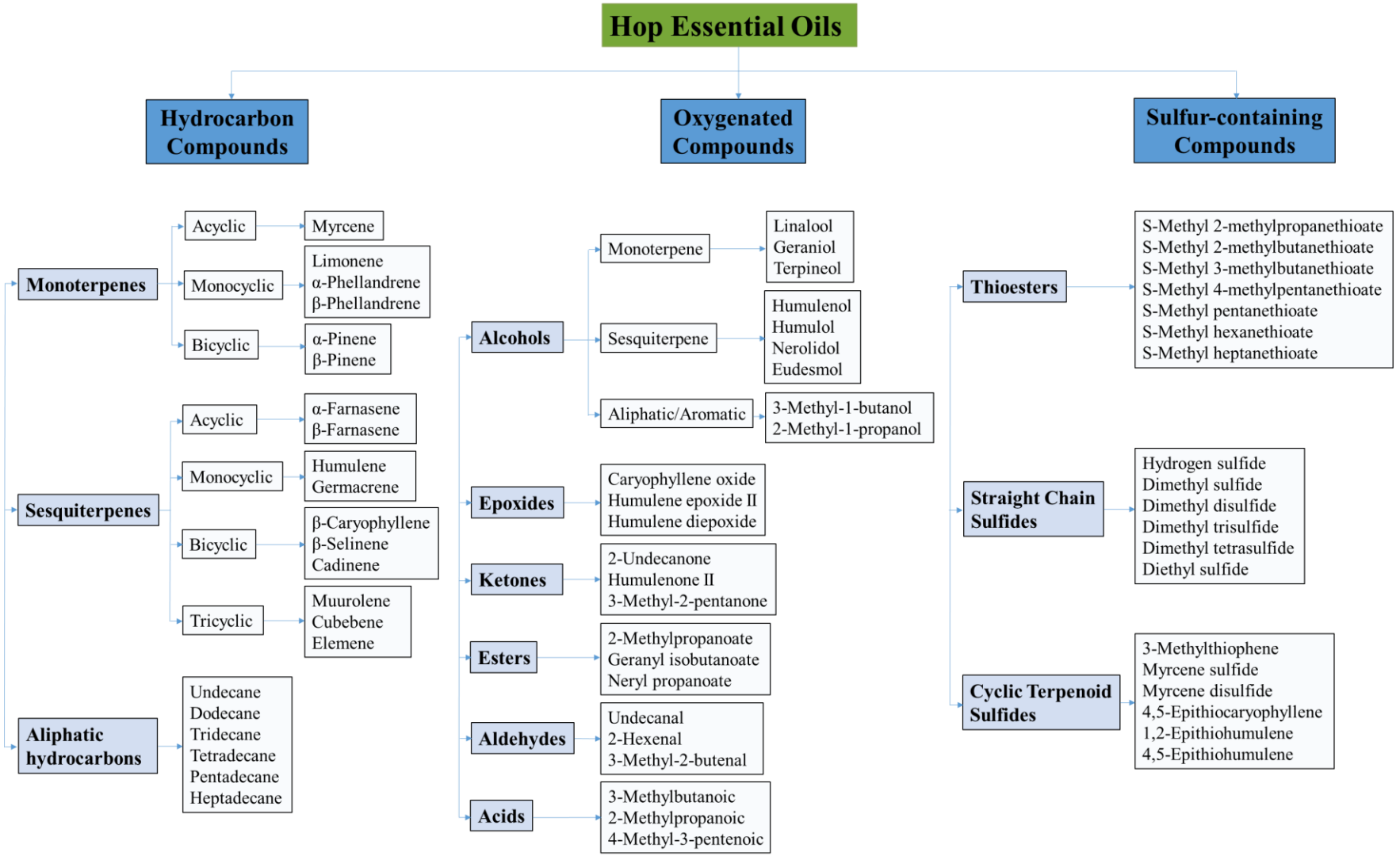

Figure 1.4 Classification of hop essential oil according to Sharpe and Laws [28].

terpene hydrocarbons myrcene, α-humulene, and β-caryophyllene typically make up the principal constituents of fresh hop oils, representing up to 80% of the total volatiles [33].

Although the oxygenated fraction is found in significantly lower amounts (represents ~30% of the total oil content) compared to the hydrocarbons, it is an extremely complex mixture composed of alcohols, epoxides, ketones, esters, aldehydes, and acids [2,28]. Autoxidation and subsequent hydrolysis of sesquiterpene hydrocarbons that occur during storage of hop result in an extensive range of reaction products, including the epoxides, with the most abundant being caryophyllene oxide and humulene epoxide II [26]. Their presence has been associated with the herbal or spicy beer character [34,35]. Sesquiterpene epoxides and diepoxides (*e.g.*, humulene diepoxide isomers) undergo further hydrolysis and rearrangements to form various alcohols and ketones [26]. The alcohols in essential oil can be subdivided into three groups, monoterpene alcohols, sesquiterpene alcohols and aliphatic/aromatic alcohols [2,28]. Monoterpene alcohols are generally biosynthetic products of myrcene, with linalool as the most abundant constituent and accompanied by geraniol, nerolidol, nerol, myrtenol and terpineol with lower amount [36,37]. Linalool is particularly important as a floral odorant in hop essential oil, and considered as an important hop aroma indicator substance in beer [26,38]. Sesquiterpene alcohols are typically hydrolysis degradation products and among many others include nerolidol, humulol, humulenol II, caryophyllenol, cadinol isomers, and eudesmol isomers [26,39].

Hop contains various sulfur-containing compounds, including methyl thioesters, thiophenes, methyl- or polysulfides, polyfunctional thiols, and sulfur adducts of

myrcene and humulene. Although sulfur compounds are present at trace levels, they may easily impact upon the overall flavour of essential oil and beer because of their potent aromas and very low odour thresholds [26,40]. A wide range of odours can be produced by these compounds, however, most of them are characterised as undesirable off-flavours and detrimental to the quality of fresh beers [2]. Of particular interest upon one sulfur compound contributing positively to hoppy beer flavour is 4-mercapto-4-methylpentan-2-one, because of its fruity blackcurrant flavour found in beers hopped with specific cultivars grown in the Australia, USA, and New Zealand [41].

It is well known that hop oil composition is strongly dependent on genetic factors, but is also affected by cultivation factors (*e.g.*, geography and ripeness) and post-harvest processing (*e.g.*, kilning and storage) [26]. It is generally agreed that the concentration of oxygenated terpenoids increases during ripening of the hop cone, hop processing and storage, but the total concentration of volatiles decreases due to evaporation and autoxidation, thereby shifting the ratio between terpene hydrocarbons and oxygenated terpenoid volatiles [33]. In addition to their remarkable aroma properties in hop and beer, terpene hydrocarbons and oxygenated terpenoids play important roles in plant physiology and ecology, such as they are potentially synthesised and emitted to defend the hop plant against herbivores and pathogens and to attract seed disseminators [42].

1.1.2 Analysis of hop aroma

With a view to decipher the mystery of hop and especially that of hop aroma, many researchers have studied hop aroma-active components. However, deeper exploration

of the volatile metabolomic patterns of natural plant products needs reliable and sensible analytical methods.

Historically, an early chemical observation of the volatile portion of hops was made by Hanin in 1819 [43]. However, only at the end of the 19th century were the first six hop aroma compounds, including myrcene and humulene, identified by Chapman [44]. Chapman already noticed, in the early days of hop oil research, that linalool and myrcene elicit the typical scent of hops. Later in 1955, fractionation of hop oil by thin-layer chromatography (TLC) and then counter-current distribution (CCD) has been reported by Rigby and Bethune, but the mixture was far more complex than the classical studies had shown [45]. It is noteworthy that precise qualitative data and systematic analysis cannot be obtained from these classical chromatographic methods and, hence, it was fortunate that about this time the technique of gas chromatography (GC) was invented and introduced by James and Martin [46,47]. Gas chromatography revolutionised the analysis of hop aromatic compounds. In 1956, Howard employed the technique and successfully separated eighteen components of oil distilled from Fuggle hops [48]. When packed GC columns gave way to capillary columns, Buttery and Ling in 1966 were able to resolve around 100 hop oil components [49]. The essential oils as well as every other natural plant product have been the main impetus to the extraordinary development of GC, and likewise the separation techniques that more than all others have contributed to the knowledge in this field [50].

GC with a flame-ionisation detector (FID) has found wide application for the analyses of hop aromatic components and provided a “crude” characterisation of various hop cultivars. Subsequently, GC coupled with other element-selective detectors, such as

sulfur-chemiluminescence (SCD) and flame-photometric (FPD) detection, were also well developed for the analyses of sulfur compounds in hop oils [33]. Modern studies have found GC combined with mass spectrometry (MS) as an indispensable tool for exploring the knowledge and science of essential oils (*e.g.*, in the areas of phytochemistry, chemotaxonomy, and biochemistry), and will be discussed further in Section 1.2. However, the response of a physical GC detector (*e.g.*, FID or MS) is not representative of odour activity, because the correlation between concentration and odour intensity may vary considerably between each component. Hence, the most abundant compound detected from a chromatographic separation may not be the most essential odorant that contribute to the overall flavour [51,52]. A more sophisticated procedure and valuable tool for the identification of character-impact odorants is GC-olfactometry (GC-O). Human assessors are employed in this technique to distinguish and evaluate volatile components when they elute from a GC column [53].

Hop essential oil is usually extracted from the hop cones or pellets prior to GC analysis, although direct headspace analysis of hop cones or pellets is also reported. A range of sample preparation methods has been applied to isolate and characterise hop oils, which include steam distillation or vacuum distillation, solvent extraction or supercritical CO_2 extraction, and headspace sampling. Particularly, automated and fast isolation techniques, e.g., direct thermal desorption (DTD) and headspace solid-phase microextraction (HS-SPME), which providing good performance for sample enrichment were applied for hop aroma analysis [33]. Differences between hop varieties can be observed by conferring distinct flavour and aroma characteristics to beer, which can be attributed to their essential oil composition and associated

phytochemistry. Therefore, chromatographic fingerprinting of hop oils has been used to interpret aroma property, identify hop variety, and determine genetic variability or diversity [54-56].

1.2 Gas chromatography – mass spectrometry

GC is one of the most well-known and successful chromatographic separation techniques utilised in the field of analytical chemistry. It has a molecular mass operating range from 2 to about 1500 Da, which is ideally suited to the chemical separation of essential oils, comprising mainly of compounds with a mass of less than 400 Da [57]. The chromatographic process starts with the introduction of vaporised analytes onto a capillary separation column (chemically coated with stationary phase) from the injector, and then is carried through the column using a flow of inert gas (such as hydrogen, helium, or nitrogen), which serves as the mobile phase. Separation is achieved by the partitioning of analytes between the inert mobile gaseous phase and immobilised stationary phase based on their relative vapour pressures and affinities for the stationary phase. The tendency of a given solute to be attracted to the stationary phase is expressed as an equilibrium constant that called the distribution constant, K_c [58]. Differences in K_c values (parameters controlled by thermodynamics) effect a chromatographic separation, which are translated into retention time differences according to the relation $K_c = k\beta$, where $k = t_R'/t_M$. A gas-phase detector (the most common of which is FID) senses the effluents from the column and provides a record of the chromatography in the form of a chromatogram, in which the peak area and height of the generated Gaussian peak are a function of the amount of solutes [58, 59].

In addition to chromatographically resolve mixtures of compounds, the GC system can provide retention data (known as retention time; t_R) which serves as complementary information for the positive identification of resolved components. Retention time is preferably converted into retention index (RI) format for improved data portability and standardisation. The commonly adopted system is developed by Kováts in 1958 for isothermal conditions, in which a mixture of two *n*-paraffins is used as the standard that are eluted before and after the analyte [60]. However, Kováts proposed his model in isothermal conditions and this in some cases might represent a limit. The most suitable method described for temperature-programmed analysis is proposed by Van den Dool and Kratz by applying the equation $RI = 100z + 100[(RT_X - RT_Z / RT_{(Z+1)} - RT_{(Z)})]$, where RT is the retention time used for the calculation, X is the solute of interest, $_Z$ and $_{Z+1}$ are *n*-alkanes with $_Z$ and $_{Z+1}$ carbon numbers, respectively [61]. It is advisable to use two columns with different stationary phase, one non-polar (*i.e.*, typically 5% phenyl polysilphenylene-siloxane) and the other polar (*i.e.*, typically polyethylene glycol), in order to enhance confidence in assignment for the identification of a given component by obtaining two RI values. Several compilations are available and widely used as references in the field of flavour and fragrance, such as those authored by Adams [62], Jennings and Shibamoto [63], and Sadtler [64], listing the RI values calculated on non-polar and/or polar columns for a number of components. However, even if these RI values are specific for a given analyte, alone they are not sufficient to be a satisfactory basis for the unambiguous component identification without at least one further validation method, such as MS [61,65].

The combination of GC with MS (GC-MS) has been demonstrated as a valuable tool

for unequivocal identification and robust quantification of plant extracts [66]. It combines the advantages of both techniques, namely, the high resolving power and the speed of GC analysis are retained, while the MS provides additional capability for qualitative analysis of unknown components (*e.g.*, providing information of elemental composition, chemical structure, and molecular weight), and quantitative analysis down to the ppb level [67]. Hence, the probability of correct compound identification increases significantly based on the providing of two independent parameters, RI values and MS library matching (comparison of experimentally acquired mass spectra with those contained in a reference MS library, such as Adams, FFNSC, and NIST) [62,68,69]. There are a large number of papers published about the analysis of plant samples (including hop essential oil) using GC-MS for metabolite profiling [70-73].

However, the complex chromatograms characterised by coeluted components and vast differences in the relative abundance of the different compounds are always expected for the metabolic profiling of plant samples. Although these issues can be partially resolved by deconvolution of the overlapping peaks (extricate MS data from overlapping chromatographic peaks to provide a pure mass spectrum of the individual compounds) [74], lacking of sufficient peak capacity (n_c) and/or stationary-phase selectivity to obtain baseline separation of all the constituents in a complex sample or of specific targeted analytes from the remaining matrices is still problematic [75]. Therefore, severe peak overlapping and the structural similarity of many isomeric compounds (*i.e.*, terpenes) particularly present in plant extract is often a hindrance for reliable MS structural elucidation. This makes accurate identification and precise quantification of compounds challenging, especially for the analysis of trace

components co-eluting with larger peaks. For example, although in the last 40 years of hop research, numerous excellent and comprehensive papers have been published, the analysis of minor volatiles (*e.g.*, thiols, aldehydes, or fatty acids) in hops remains challenging. The analytical challenges primarily relate to the factors of 1) hop aroma compounds differ in their chemical and physical properties (*i.e.*, reactivity (functional groups), polarity, boiling point, and molecular weight); 2) the concentration of individual volatiles varies greatly, starting from sub-ppb levels; 3) the flavour activity of compounds varies over several orders of magnitude; 4) there is no brief list of compounds which are responsible for hop aroma and hoppy beer flavour [33]. Thus the desire to achieve enhanced selectivity and specificity drives the development of more advanced analytical technologies (*i.e.*, higher separation order GC and/or MS technologies).

1.3 Multidimensional gas chromatography

1.3.1 Definition of multidimensional chromatography

Due to the inability of one-dimensional (1D) chromatography to completely separate complex samples, scientists started to explore the possibilities of multidimensional separation methods. Although the first example of multidimensional chromatography (MDC) date back to planar chromatography in 1944 with the elution of two mobile phases in orthogonal directions [76], it was the introduction of the multidimensional method concept in 1984 by Giddings which gave a true insight of the new perspectives for the separation sciences [77]. According to the definitions proposed by Giddings, there are two prerequisites to produce them: 1) components be subjected to two or

more largely independent separative displacements; 2) the separation must be structured such that whenever two components are adequately resolved in any one displacement step, they will generally remain resolved throughout the process [78]. By implementing distinct separation mechanisms on each dimension, the possibility of processing samples according to their different physicochemical properties to resolve a maximum number of solutes can be achieved.

There are two major type of MDC techniques, namely heart-cutting MDC (H/C MDC) and comprehensive MDC. H/C MDC enables the transfer of a single or few selected chromatographic bands of overlapping components from a primary chromatographic column to a secondary chromatographic column of different selectivity for additional separation. The peak capacity for H/C MDC is equal to the sum of the n_c of the first and second dimensions, in which the latter is multiplied by the number (x) of heart cuts; $[n_{c1} + (n_{c2} \times x)]$ [75]. For sample that requires comprehensive analysis in two different dimensions, a multidimensional comprehensive chromatography (MDCC) technique is required. The total peak capacity for an ideal MDCC system is equal to the multiplication of the n_c of the first and second dimensions ($n_{c1} \times n_{c2}$) [75,78]. Thus, a well implemented MDC approach permits a means for increasing the peak capacity, and thus the resolution of more components in complex mixtures. These principles and benefits of MDC were also well applied within the GC community, the two primary approaches of MDGC involve conventional heart-cut multidimensional GC (H/C MDGC) and comprehensive two-dimensional GC (GC×GC), which will be discussed in the following sections.

1.3.2 Heart-cut multidimensional gas chromatography

Since the first demonstration of H/C MDGC was described by Simmons and Snyder in 1958 for analysis of a stabilised platformate sample [79], it has been widely used for enhanced separation of target compounds or regions of interest by transferring from a primary column (first-dimension; ^{1}D) to a secondary column (second-dimension; ^{2}D), which is of different stationary phase, and hence providing two independent separation mechanism [80-82]. Therefore, effective combination of discrete dimensions generates greater peak capacity for isolation of target analytes, from interfering compounds, was achieved by employing more than one chemical selectivity.

In H/C MDGC, a heart-cut represents a small portion of effluent (generally containing unresolved or poorly resolved target compounds) from the ^{1}D column, which is directed into either a ^{2}D column or a detector module. Transfer of chromatographic bands between the two dimensions was typically achieved with either a mechanical switching valve, or using a pressure-driven switching device (Deans switch) [83,84]. With the invention of Deans switch at the end of the 1960s, Deans' basic principle of pressure switching has been used extensively for implementing H/C operations. This switching device has provided some unquestionable advantages, such as no artifacts formation, temperature limitations, memory effects, and low contribution towards peak broadening [80]. Based on the Deans switch mechanism, there are several commercially available switching designs for performing H/C MDGC analysis including Trajan (SilFlow microchannel device), Agilent (capillary flow technology, CFT), Gerstel (multi-column switching, MCS), PerkinElmer (Swafer micro-channel flow technology), Shimadzu (multi Deans switching system technology, MDSS), and

Brechbühler scientific (moving capillary stream switching, MCSS) [83]. Meanwhile, two major requirements need to be fulfilled for the aforementioned devices: 1) narrow channels in the device to provide minimal peak dispersion; 2) low thermal mass compatible with fast thermal program [83]. Their advantages and disadvantages have been reviewed and compared thoroughly in literature [83,85].

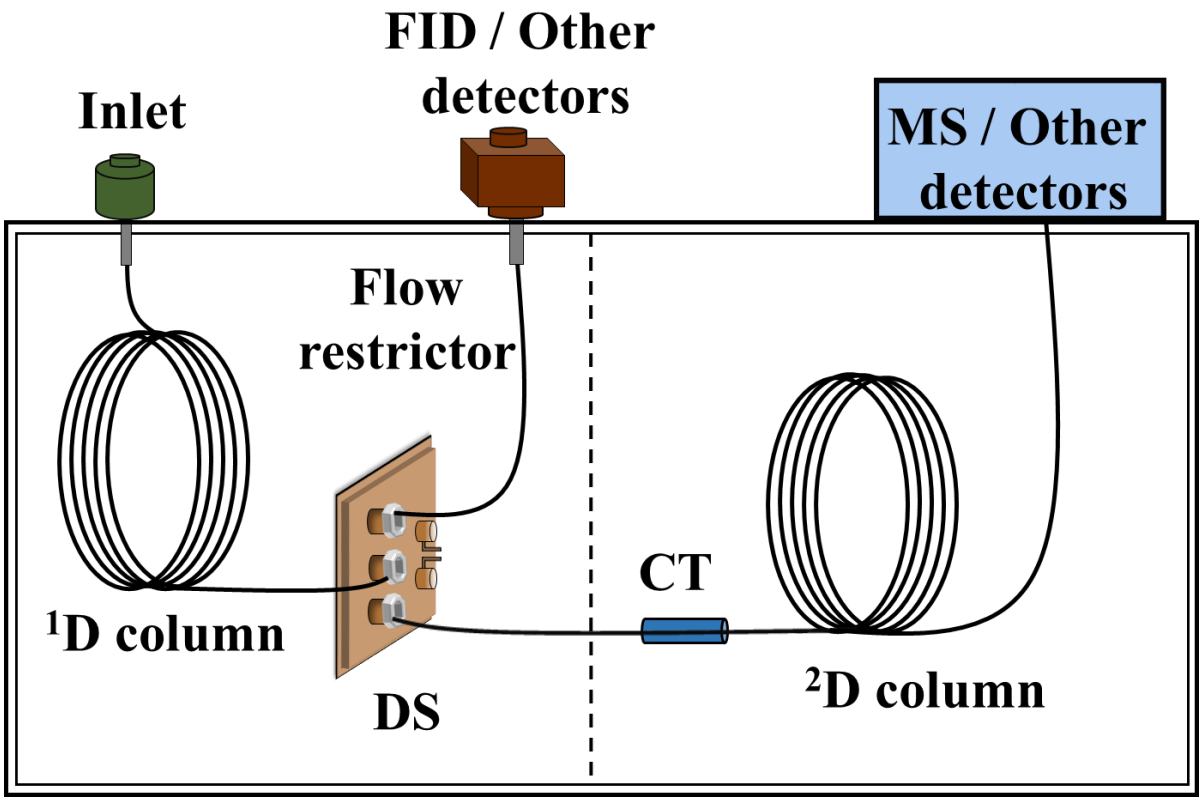

Figure 1.5 Schematic diagram of a typical H/C MDGC system. The second oven (indicated by dotted line) is optional. DS, CFT Deans switch device; CT, cryogenic trapping device.

Various experimental configurations existing for MDGC analysis, such as pressure tuning 2D-GC, flow switching MDGC, stop-flow fractionated MDGC, multiple heart-cut & cold-trap MDGC, and multiple parallel-2D GC, etc. [85], which provides analyst a great deal of flexibility in designing a method for increasing the separation

power of a chromatographic system. **Figure 1.5** depicts a schematic diagram of a typical H/C MDGC configuration. Two capillary columns (*e.g.*, 30 m × 0.25 mm I.D. × 0.25 μm d_f) are connected in series and characterised by a differing selectivity (*e.g.*, nonpolar-polar, polar-enantioselective, etc.). A dual-oven system is preferred to offer greater flexibility by having independent temperature control for both dimensions. Components without further analytical interest are delivered to a monitor detector, which normally employ a FID and/or other universal detectors. A cryogenic trapping (CT) device can be chosen to place at the inlet of the ^{2}D column to trap and refocus the H/C region prior to ^{2}D separation. The CT approach is an option bringing the following advantages: 1) minimising the dispersion effect which arising from the ^{1}D column; 2) maximising the ^{2}D column efficiency through analyte refocusing; 3) peak capacity increase, by substantial reduction of the width of ^{1}D chromatography bands; 4) reducing the possibility of intermingling two or multiple successive H/C fractions in the ^{2}D column; 5) collection of volatile effluents in preparative GC system [86-89].

1.3.3 Heart-cut multidimensional gas chromatography for targeted analysis

It is clear that MDGC has been best suited for applying maximum resolving power to a targeted heart-cut region (normally the complex or unresolved portion of the sample) of the chromatogram. Therefore, MDGC approaches have gained acceptance as a useful tool for the identification and/or quantification of target components in complex matrixes, due to the obtained better separation (greater peak capacity and enhanced resolution) after chromatographic pre-separation over traditional 1D-GC analysis. A wide range of applications in various fields, such as but not limited to the following

categories: forensic and biological, food and flavours/fragrances, environmental and agricultural, and petroleum have invariably employed MDGC technology [90]. One of the most active areas for MDGC approaches has been the analysis of plant samples (*e.g.*, essential oil). The metabolic compositions in natural plant extracts are of such diversity and complexity that always not be anticipated by analysts, hence, the peak capacity of 1D GC separation will often be exceeded. Different column combinations (*e.g.*, nonpolar-polar, polar-enantioselective) and detection (*e.g.*, FID/MS, MS/olfactometry) methods have been implemented according to the chemical properties of sample components and the aim of analysis [85].

Employing an enantioselective phase (*e.g.*, enantioselective column) as the ^{2}D column in the MDGC analysis, namely enantioselective-MDGC (*enantio*-MDGC), has been extensively utilised for the analysis of optically active compounds. The enantiomeric distribution of selected chiral terpenic compounds can be beneficial for identifying adulterated foods, beverages, and plant-derived products (*e.g.*, essential oils), for authentication or manufacture monitoring [91]. In a typical *enantio*-MDGC system, a selected region(s) (containing the targeted chiral compounds) from the non-enantioselective ^{1}D column is H/C into the enantioselective ^{2}D column to achieve the enantiomeric separations, providing an accurate determination of the enantiomeric fraction and enantiomeric ratio of the selected chiral compounds. In the area of flavour analysis, the GC-olfactometry (GC-O) technique is used to detect and distinguish individual odour-active compound(s) in a range of aroma extracts through sniffing of the chromatographic effluent from a GC column outlet. Due to the requirement of high resolution approaches for the analysis of the odour complexity in volatile mixtures,

MDGC connected with oflactometry (MDGC-O) was introduced to provide improved knowledge of aroma composition in food/flavour samples [92]. The most common applications of MDGC-O are wine and essential oil analysis. Selected MDGC applications describing analysis of plant-derived samples (mainly focused on essential oils) are summarised in **Table 1.1**. The main weakness of the MDGC method is that the H/C fractions have to be a small portion of the total sample (targeted analysis), since heart-cutting long duration of effluents would cause the loss of resolution obtained from the ^{1}D column, and increase the potential of recombining transferred peaks that were previously separated during the ^{1}D separation. Therefore this MD technique is not suitable when many peaks are of interest, and particularly when they are scattered throughout the ^{1}D separation [85]. Alternatively, for full characterisation (*i.e*., for non-targeted analysis) of a complex mixture, GC×GC uses continuous heart-cutting with a specific sampling period for non-discriminative analysis should be considered.

Table 1.1 Selected applications of MDGC approaches for analysis of plant-derived samples.

Sample	Research objectives	Analytical approach	Column types	Interface	Ref
Peppermint & lavender oil	Method development for online enrichment of target compounds	MDGC-FID	^{1}D: SLB-5ms (15 m×0.32 mm I.D.×1.00 µm d_f) ^{2}D: SLB-IL100 (30 m×0.25 mm I.D.×0.20 µm d_f) ^{1}D: DB-FFAP (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: DB-1301 (10 m×0.18 mm I.D.×0.40 µm d_f)	Deans switch (Agilent Technologies)	[88]
Hop essential oil	Comparison of odour active compounds in the spicy fraction of hop samples	MDGC-O/FID	^{1}D: HP-5 (30 m×0.32 mm I.D.×0.25 µm d_f) ^{2}D: Solgel Wax (30 m×0.32 mm I.D.×0.5 µm d_f)	Deans switch (Agilent Technologies)	[93]
Sandalwood oil	Determination of the santalol fraction in a range of Australian sandalwood oils of different origin	MDGC-FID/MS	^{1}D: SLB-5ms (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: Supelcowax (30 m×0.25 mm I.D.×0.25 µm d_f)	MDS (Shimadzu)	[94]
Australian tea tree oil	Fast enantioseparation of three key chiral monoterpenes for authenticity control	Fast enantioselective MDGC-MS	^{1}D: Rxi®-17Sil MS (30 m×0.25 mm I.D.×0.25 µm d_f)	Deans switch (Agilent Technologies)	[95]

			^{2}D: AstecCHIRALDEX B-PM (30 m×0.25 mm I.D.×0.12 µm d_f)		
Lime essential oils	Determination of the enantiomeric distribution of chiral compounds for authenticity assessment	Enantioselective MDGC-MS GC-C-IRMS	^{1}D: SLB-5ms (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: Megadex DETTBS-β (25 m×0.25 mm I.D.×0.25 µm d_f)	Deans switch (N/A)	[96]
Limonene and carvone plant/essential oil	Chiral analysis of both solid and liquid sample by direct introduction to MDGC	Enantioselective MDGC-MS	^{1}D: ZB-Wax (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: Chirasil-β-Dex (30 m×0.25 mm I.D.×0.25 µm d_f)	Deans switching system (Varian)	[97]
Lemon and mandarin petitgrain oil	Investigation of the enantiomeric distribution of selected chiral compounds	Enantioselective MDGC-MS GC-C-IRMS	^{1}D: SLB-5ms (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: Megadex DETTBS-β (25 m×0.25 mm I.D.×0.25 µm d_f)	N/A	[98]
Lavender oil	Rapid sequential separation and identification of essential oil compounds using continuous MDGC	Rapid repetitive MDGC-MS	^{1}D: DB-5 (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: DB-Wax (7 m×0.10 mm I.D.×0.10 µm d_f)	LMCS (Chromatography Concepts Ltd)	[99]

Mandarin oil	Analysis of volatile chiral components in natural Italian cold-pressed mandarin essential oils	Enantioselective MDGC-MS	^{1}D: SLB-5ms (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: Megadex DETTBS-β (25 m×0.25 mm I.D.×0.25 µm d_f)	MDSS (Shimadzu)	[100]
Gin essential oil mixtures	Identification of organoleptic constituents of gin	MDGC-MS	^{1}D: Supelcowax-10 (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: HP5-MS (30 m×0.25 mm I.D.×0.25 µm d_f)	Deans switch (Agilent Technologies)	[101]
Hop essential oil	Investigation of the sweat-like odorous volatile compounds	MDGC-MS	^{1}D: TC-Wax (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: InertCap 1 (60 m×0.25 mm I.D.×0.25 µm d_f)	MCS 2 (Gerstel)	[102]
Coriander leaf and hop essential oil	Identification of character-impact odorants	MDGC-FID/O	^{1}D: HP-5 (30 m×0.32 mm I.D.×0.25 µm d_f) ^{2}D: Solgel Wax (30 m×0.32 mm I.D.×0.5 µm d_f) ^{1}D: HP-5 (30 m×0.32 mm I.D.×0.25 µm d_f) ^{2}D: Solgel Wax (30 m×0.25 mm I.D.×0.25 µm d_f)	Deans switch (Agilent Technologies)	[103]

Tanacetum L. essential oil	Investigation of composition and enantiomeric distribution	MDGC-MS	^{1}D: HP-Innowax (60 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: OV 1701 (25 m×0.25 mm I.D.×N/A)	MCS (Gerstel)	[104]

1.3.4 Comprehensive two-dimensional gas chromatography

Introduction to GC×GC

GC×GC, essentially an extended version of conventional heart-cut MDGC, was introduced and pioneered by Liu and Phillips in the early 1990s [105] to fulfil the unrelenting requirement of resolving (identifiably and quantifiably separate) more compounds in highly complex matrices. In contrast to MDGC, GC×GC is a continuous sampling process in which the entire effluent from the primary separation system (^{1}D column) is subjected to the secondary separation system (^{2}D column) via an interface while preserving the resolution of ^{1}D throughout the analysis. Hence the so-called orthogonal separation conditions were created through two GC separations with fundamentally different separation mechanisms that are applied to the entire sample [106]. The most important outcome of GC×GC has been the great increase in the peak capacity, which is ideally equal to the product of the peak capacities ($n_{c1} \times n_{c2}$) in each dimension as described by Giddings [78]. Blumberg and coworkers have theoretically demonstrated the possibility of obtaining an order of magnitude peak capacity gain by GC×GC system [107]. Although such claimed value is probably an excessive estimation of the real value, the practice peak capacity obtained is unprecedented and makes the method most appropriate to unravel a multitude of volatile or semi-volatile samples [108]. Specifically, the major advantages of GC×GC, compare to traditional 1D GC methods, are characterised by: 1) increased selectivity (different selectivities provided by the employed two stationary phases) and resolving power; 2) enhanced sensitivity (a band compression effect generated through cryogenically modulated applications); 3) increased identification power due to the

orderly distributions of compounds in the 2D separation space (*i.e.*, homologous compounds) [108,109].

Implementation of GC×GC

The generic instrumental set-up of GC×GC consists of two columns, a transfer interface (generically defined as a modulator) and the option for either single or dual column-oven operation, as shown in **Figure 1.6**. The modulator interface is arguably the most important component of any GC×GC system. The role of the modulator is to accumulate or trap, possibly re-concentrate (in cryogenic systems), and re-inject portions (heart cuts) of the ^{1}D effluent into the ^{2}D as a repetitive series of pulses continuously throughout the analysis [85]. The modulator must be capable of precisely and rapidly generating a continuous series of narrow ^{2}D injection bandwidths, which are critical for maximising the separation performance of the ^{2}D column, and while still preserving the integrity of the ^{1}D separation [105]. Another key requirement for implementing GC×GC analysis is that the ^{2}D separations should be finished within the defined modulation period (P_M) to minimise the occurrence of wrap-around [109]. The term modulation ratio (M_R) defines the number of modulations across the ^{1}D peak, proposed as $M_R = w_b/P_M = (w_h \times 1.6985)/P_M$, where w_b and w_h are the ^{1}D peak base width and ^{1}D peak width at half-height, respectively. For precise quantification of trace compounds, an M_R of at least 3 is recommended, whilst an M_R of approximately 1.5 is sufficient for semi-quantitative analysis [110].

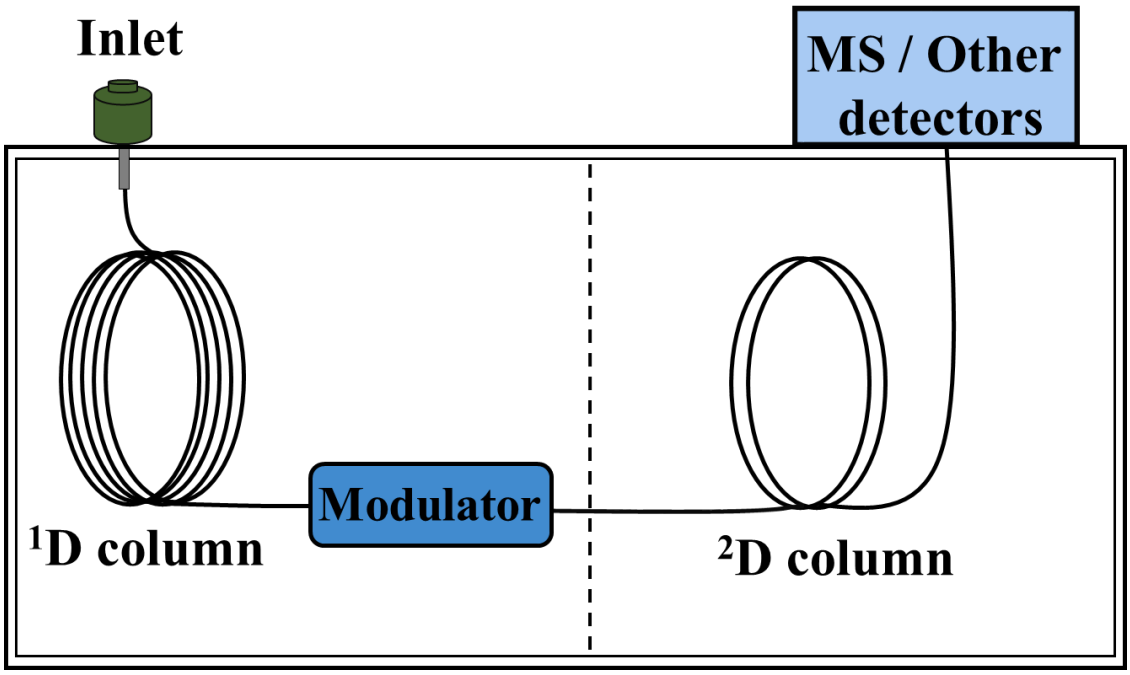

Figure 1.6 Schematic diagram of a typical GC×GC system. The second oven (indicated by dotted line) is optional.

Modulator

Technically, modulation devices can be divided into two main types, namely thermal modulators (*e.g.*, heater-based and cryogenic modulators) and valve-based modulators (*e.g.*, diaphragm valves and flow modulation) [111,112]. Thermal modulators operate based on low thermal temperatures to trap and focus the ^{1}D effluent, followed by rapid introduction of the effluent to the ^{2}D column via rapid heating. Typically, there are three types of thermal modulators, namely, heated sweeper, resistively heated trap, and cryogenic focus, which can be further divided into jet trap and longitude movable trap [113]. The most frequently used cryogenic based modulator is the jet trap, which uses cryogenic gas (*i.e.*, liquid nitrogen or liquid carbon dioxide) to trap, focus, and reinject the ^{1}D effluents to the ^{2}D column. Valve-based modulators collect the ^{1}D effluent

using a short collection loop, which is then quickly flushed and switched onto the ^{2}D column with high flow rates (*e.g.*, 15 mL/min). The first valve-based modulator was based on a fast-switching diaphragm valve, but it has mostly been replaced by flow modulation, which is the only one commercially available valve-based modulators constructed with a capillary flow technology [113]. Overall, all modulators developed have distinct advantages and disadvantages, and the development of different modulation principles and their applications have been extensively reviewed [90,112-114].

Column dimensions and phases

In order to achieve orthogonal separation in a GC×GC system, it is necessary to employ column combinations that provide independent separation mechanisms in the ^{1}D and ^{2}D [115]. The most commonly used column set has been a nonpolar ^{1}D column and polar ^{2}D column [116]. In such combination, the solutes mainly interact as a function of boiling point (dispersion force) in the ^{1}D, while in the ^{2}D as a function of activity coefficients (*i.e.*, steric effects, π–π interaction, hydrogen bonding, etc.) of the solutes [117]. The main benefit of orthogonality is that ordered structures are displayed in the GC×GC chromatogram for samples containing structurally related compounds (*i.e.*, isomers, congeners, and homologues). Such structured 2D chromatograms have been treated as a powerful analyte identification tool (*i.e.*, group-type identification), since the related compounds were displayed as clusters or bands [115]. Although the analytes' chemical structure cannot be decoded directly from the structured peak pattern, which still be characterised as a unique 2D signature of the analytes. But for some applications, a polar ^{1}D and nonpolar ^{2}D column combination

may also be beneficial of the better occupation of the 2D separation space, and/or showing highly interesting structural distributions for a range of compounds with different polarities [118,119]. Since the technique of GC×GC depends on fast analysis of pulsed bands, the ^{2}D column must be a fast elution column to achieve complete ^{2}D separation in a few seconds. Therefore, a short, narrow-bore, thin-film column is commonly used in the ^{2}D, typically 0.5–2 m × 0.1 mm I.D. This ensures that the minimisation of wrap-around and maintaining high resolution in the ^{2}D separation space can be attained. Inversely, the dimension of the ^{1}D column is less important, instead it is more critical to consider the peak width generated by the ^{1}D separation. Thus a conventional ^{1}D column similar to those routinely employed in 1D-GC system is normally used, *e.g.*, 25–30 m × 0.25 mm I.D. × 0.25 μm d_f [120].

Detector

Detection is the other critical part of a GC×GC system. The excellent separation power of GC×GC produces very narrow peaks with widths of, generally, 50–600 ms at the baseline [121]. Such rapid chromatography bands require detectors characterised by high acquisition frequencies (minimum 50 Hz), low time constants, and small internal volumes. These detector characteristics are essential to obtain a reliable and accurate peak reconstruction (10 data points per peak would be preferable) [122]. A small internal volume is also required to substantially limit the dispersion generated by the detector. The FID has been the most widely used detector for universal detection due to its negligible internal volume and high acquisition frequency ranging from 50–300 Hz [122]. Other element-selective detectors such as atomic emission (AED), micro-electron capture (μECD), nitrogen-phosphorous (NPD), flame-photometric (FPD),

sulfur-chemiluminescence (SCD), and nitrogen-chemiluminescence (NCD) detectors have also been used for selective applications in GC×GC analysis [123].

Although the aforementioned detectors provide peak recognition, no structural information can be offered. Hence, the combination of a spectrometric detector, specifically MS, is highly attractive for allowing the identification of the numerous separated components. The main requirement for this hyphenation technique of GC×GC-MS is sufficiently fast acquisition frequency. This acquisition speed limits the use of quadrupole MS (qMS), nevertheless some studies resolved this problem by using a reduced mass-scan range to obtain adequate acquisition speed in GC×GC analysis [124,125]. Time-of-flight mass spectrometry (TOFMS) has demonstrated its excellent compatibility for GC×GC system due to its capability of providing fast acquisition speed (up to 500 spectra/s), and instantaneous measurement of all masses for every pulse of ions [120,125,126]. In addition, the non-scanning character of the TOF process has the advantage of producing non-skewed mass spectra (spectral continuity) because virtually all ions are collected at the same time point of the chromatogram. This spectral continuity allows optimal performance of deconvolution algorithms on overlapped spectra, and provides a higher mass accuracy [126]. The advantages of GC×GC-MS include the general GC×GC benefits such as unprecedented selectivity (three separation dimensions, correlated with polarity, volatility, and mass), improved separation (increased n_c), high sensitivity (band compression generated by using cryogenic gases), and structured 2D chromatograms undoubtedly assist the qualitative and/or quantitative analyses of a wide dynamic range of complex samples [108,127].

Data presentation

Acquisition, visualisation, and processing of data in comprehensive two-dimensional chromatography were once the Achilles heel of the comprehensively coupled separation approaches. Fortunately, there has been continuous development of software to enable work in this field. The raw data obtained can be readily converted into a 2D contour plot, or a 3D plot with two retention times (x- and y-axes) and signal intensity as the z-axis by using dedicated software packages (*e.g.*, GC Image, ChromaTOF, and ChromSquare, etc.) [113]. Moreover, interpreting the 2D data by employing advanced chemometrics can provide greater value to a GC×GC study. Since chemometric tools may substantially increase the possibility of disclosing the 'hidden information' in higher-order data structure obtained from a GC×GC separation, and taking full advantage of the GC×GC technique [85,128]. Hence, chemometric interpretation has been treated as a perfect accompaniment to GC×GC data processing, with continuous applications to areas such as profiling and/or differentiation of volatile chemical patterns in food and beverage, and metabolomics [85,129,130].

1.3.5 Comprehensive two-dimensional gas chromatography for untargeted analysis

Advantages and separation performances achieved with GC×GC have greatly enabled analysts to expand the chemical maps (namely, untargeted analysis or fingerprinting) of a variety of complex volatile or semi-volatile samples, such as food, flavour and fragrance, environmental, biological, and petrochemicals [90,113]. Despite the wide range of applications, GC×GC was not used for plant extracts analysis until the first

description of a qualitative comparison of spearmint and peppermint essential oils reported in 2000 [131]. Since then, a variety of investigations have highlighted the important role of this technology for plant-derived samples including essential oil applications, where not only a much broader view of the whole phytochemical properties but also the opportunity to discover novel and/or previously unknown plant secondary compounds were provided [132,133].

In general, for plant samples of very complex composition, GC×GC can be employed for mainly three analytical scopes: 1) targeted analysis (identification and/or quantification of known components); 2) untargeted analysis (comprehensive qualitative screening); 3) fingerprinting analysis (chemical profiling and/or differentiation) [134,135]. Specifically, GC×GC fingerprinting, a form of untargeted analysis, can be used to investigate the similarities and/or differences of plant-derived samples on the basis of specific technological treatments (*i.e.*, type of roasting), geographical origin, and sensorial properties (*i.e.*, cultivar of hops) [132,136].

The high diversity of natural plant mixtures has motivated analysts to evaluate unique sampling approaches and different column sets for various GC×GC applications. In addition to the typical column combinations, such as apolar-polar and polar-apolar, the use of an enantioselective column in GC×GC arrangement for the enantioselective analysis of essential oil has also been reported [137,138]. There are two distinctly different means to implementing the enantioselective GC×GC experiment, namely *enantio*-GC×GC (with a ^{1}D enantioselective column) and GC×*enantio*-GC (with a short ^{2}D enantioselective column) [133]. The main advantage of *enantio*-GC×GC is the reduced total analysis time over a method requiring multiple heart-cuts. Obtaining

a high degree of ^{1}D resolution for the chiral compounds of interest is the most crucial requirement to producing a successful result [137]. The implementation of GC×*enantio*-GC is more demanding than *enantio*-GC×GC, most likely because of the difficulties of obtaining satisfactory resolution of specific enantiomers on a very short ^{2}D column [132,133,138]. However, the use of *enantio*-MDGC shows to be the most appropriate option to prevent co-elution between targeted enantiomers and matrices [132]. Similarly, the combination of GC×GC and olfactometry (GC×GC-O) is also technically demanding, mainly due to the mismatch between the very narrow ^{2}D peak (*i.e.*, 100–500 ms) and the breathing cycle of a human (*i.e.*, 3–4 s) [139]. So the application of H/C MDGC-O for the study of aroma-impact regions remains the best option for plant analysts. Selected studies of plant-derived samples (mainly focus on essential oils) using GC×GC approach are summarised in **Table 1.2**. It is noted that GC×GC has extended the analytical efficiency to a more substantial molecular coverage, with the most significant advantage of providing much greater capacity for resolving constituents in the presence of complex matrices [81].

Table 1.2 Selected applications of GC×GC approaches for analysis of plant-derived samples.

Sample	Research objectives	Column types	Modulator	Detector	Ref
Olive oil	Combined untargeted and targeted fingerprinting method for the analysis of VOCs distributions and ripening indicators	^{1}D: SolGel Wax (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: OV1701 (1 m×0.10 mm I.D.×0.10 µm d_f)	Two-stage KT 2004 loop thermal modulator (Zoex Corporation)	MS	[134]
Hop oil volatiles	Characterisation of novel varietal oxygenated sesquiterpenoid fractions	^{1}D: Rtx-pona (50 m×0.25 mm I.D.×0.5 µm d_f) ^{2}D: BPX-50 (2 m×0.15 mm I.D.×0.15 µm d_f)	Dual jet cryogenic modulator (Thermo Fisher Scientific)	TOFMS	[140]
Rosa damascena Miller essential oils	Enantioselective separation of terpenoic solutes for quality or authenticity control	^{1}D: Rt-βDEXse (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: HP INNOWax (5 m×0.25 mm I.D.×0.15 µm d_f)	Flow modulator (Agilent Technologies)	FID, QMS	[141]
Virgin olive oil	Defining chemical blueprint of volatiles to be correlated to the product sensory quality	^{1}D: Rxi-5ms (30 m×0.25 mm I.D.×0.50 µm d_f) ^{2}D: Supelcowax-10 (1.2 m×0.10 mm I.D.×0.10 µm d_f)	Loop-type single-jet cryogenic modulator	QMS	[142]

		^{1}D: SolGel Wax (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: OV1701 (1 m×0.10 mm I.D.×0.10 µm d_f)	Two-stage KT 2004 loop thermal modulator (Zoex Corporation)	MS	
Eucalyptus spp. leaf oils	Untargeted metabolic profiling of secondary metabolites using generalised processes for components identification	^{1}D: SUPELCOWAX®10 (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: Rxi®-5Sil MS (1 m×0.10 mm I.D.×0.10 µm d_f)	LMCS (Chromatography Concepts Ltd)	Q-TOFMS	[143]
Vetiver essential oils	Qualitative and quantitative analysis of volatile constituents	^{1}D: VF-5MS (30 m×0.25 mm I.D.×0.20 µm d_f) ^{2}D: DB-Wax (1.25 m×0.10 mm I.D.×0.1 µm d_f)	Dual jet, loop-type cryogenic modulator (Zoex Corporation)	FID, MS	[144]
Piper regnellii (Miq.) C.DC. essential oils	Chemical characterisation of volatile components	^{1}D: DB-5 (60 m×0.25 mm I.D.×0.10 µm d_f) ^{2}D: DB-17 ms (2.15 m×0.18 mm I.D.×0.18 µm d_f)	Dual-stage, quad-jet cryogenic modulator (LECO)	TOFMS	[145]
Mandarin & spearmint essential oils	Untargeted and targeted characterisation of complex samples	^{1}D: SLB-5ms (11.75 m×0.1 mm I.D.×0.10 µm d_f) ^{2}D: IL-60 (5 m×0.25 mm I.D.×0.25 µm d_f)	Seven-port wafer chip modulator (SGE)	QqQMS	[146]

Rosemary essential oils	Determination of terpene compositions in the aroma fraction	^{1}D: HP-5MS (30 m×0.25 mm I.D.×0.25 μm d_f) ^{2}D: DB-17MS (5 m×0.25 mm I.D.×0.25 μm d_f)	Valve modulator	FID, MS	[147]
Agarwood oils	Metabolic profiling of plant-fungus interaction	^{1}D: SUPELCOWAX®10 (30 m×0.25 mm I.D.×0.25 μm d_f) ^{2}D: Rxi®-5Sil MS (1 m×0.10 mm I.D.×0.10 μm d_f)	LMCS (Chromatography Concepts Ltd)	Q-TOFMS	[148]
Rosewood leaf essential oil	Chemical characterisation of essential oil profiles	^{1}D: HP-5 (30 m×0.25 mm I.D.×0.25 μm d_f) ^{2}D: DB-Wax (1 m×0.10 mm I.D.×0.10 μm d_f)	Home-made four-jet cryogenic modulator	QMS	[149]
Essential oil of perfume & rosemary	Quantitative analysis of volatiles in essential oils using multivariate curve resolution	^{1}D: HP-5 (30 m×0.25 mm I.D.×0.25 μm d_f) ^{2}D: Supelcowax-10 (1 m×0.10 mm I.D.×0.10 μm d_f)	Lab-made four jet cryogenic modulator	FID	[150]
Mentha haplocalyx essential oils	Phytocharacterisation and routine identification of target compounds and enantiomers	^{1}D: DB-XLB (30 m×0.25 mm I.D.×0.25 μm d_f) ^{2}D: BPX-50 (1 m×0.10 mm I.D.×0.10 μm d_f)	ZX2 thermal modulator (Zoex Corporation)	HR-TOFMS	[151]

Mentha × *piperita* L. essential oils	Quantitative profiling and fingerprinting of essential oils of different complexity	^{1}D: SE52 (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: OV1701 (5 m×0.25 mm I.D.×0.25 µm d_f)	Reverse-inject differential flow modulator	QMS	[152]
Piper amalago essential oils	Phytocharacterisation of the chemical composition for antilithiasic activity and acute toxicity study	^{1}D: DB-5 (60 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: DB-17 (2.15 m×0.18 mm I.D.×0.18 µm d_f)	Loop-type cryogenic modulator (Zoex Corporation)	QMS	[153]

1.4 Hybrid system designs

Despite obtaining a peak capacity approximately the multiplication of that of the participating columns, it was pointed out that GC×GC is still unable to provide complete separation (ideally as fully resolved single components). Unambiguous assignment of all target analytes in very complex samples is not always possible. Hence, development of higher order GC designs with expanded resolving power, able to cater for a multitude of chemical compounds of widely different abundance and physicochemical properties, is desirable.

With the intervention of a variety of GC ancillary devices that enable highly precise flow switching, effluent modulation, cryotrapping, and multiple columns interfaced in a facile manner, an extensive array of new operational procedures for sophisticated gas phase chromatographic analysis can be introduced [81]. Several recent studies have described advanced multidimensional GC designs comprising more than two dimensions of GC separation (incorporating three or more GC capillary columns), which permit a range of new separation procedures and ultrahigh resolution analysis. These include the recently proposed multi-column GC×GC, integrated/switchable MDGC with GC×GC, comprehensive three-dimensional (3D) GC, smart 3D micro-GC, preparative 3D GC, hybrid (sequential) GC×GC-MDGC, and enantioselective four-dimensional (4D) dynamic GC as examples of higher order GC designs that have moved beyond conceptualization and implementation, to further enhance separation selectivity in ways not possible in 1D and 2D GC techniques [154-162]. Furthermore, various other multiple coupled systems, such as combinations of liquid chromatography (LC), or supercritical fluid chromatography (SFC) to GC, allow

efficient chemical analysis of a broad range of components [163-165]. All these designs are directed to the goals of either providing improved analysis of either global (*i.e.*, expanding the metabolite coverage for metabolomics analysis) or target detection and/or measurement of analytes. Selected recent applications of these advanced designs of hybrid systems for volatiles analysis are summarised in **Table 1.3**. Unquestionably, by judicious choice of implementing these innovative multidimensional separation systems, better understanding of the essential chemical information related to the very complex real-world samples (*e.g.*, their total components and physicochemical properties), and substantially improved capability for absolute identification may be realised [81].

Table 1.3 Selected applications of advanced hybrid GC systems for volatiles analysis.

Sample	Research objectives	Proposed technique	Column types	Ref
Volatile organic compounds	Evaluation of a multiplexed system for complex sample analysis	Multiplexed dual-secondary column GC×GC system GC×2GC-FID	^{1}D: Rtx-624 (10 m×0.25 mm I.D.×1.4 µm d_f) ^{2}D$_1$: Rtx-Wax (1.0 m×0.15 mm I.D.×0.15 µm d_f) ^{2}D$_2$: Rtx-200 (1.0 m×0.15 mm I.D.×0.15 µm d_f)	[154]
Australian tea tree essential oil	Evaluation of a multiplexed system for improved compound identification	Multiplexed dual-primary column GC×GC system 2GC×GC-MS	^{1}D$_1$: DB-1 (30 m×0.25 mm I.D.×0.25 µm d_f) ^{1}D$_2$: DB-Wax (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: Rxi-17SilMS (0.45 m×0.15 mm I.D.)	[155]
Shiraz wine	Evaluation of an integrated separation system for high resolution profiling of aroma sample	Integrated MDGC/GC×GC system with FID/MS/olfactometry detection	^{1}D: DB-FFAP (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D$_S$: BPX5 (0.9 m×0.10 mm I.D.×0.10 µm d_f) ^{2}D$_L$: DB-5 ms (30 m×0.25 mm I.D.×0.25 µm d_f)	[156]
Diesel fuel	High resolution chemical characterisation of complex sample	Comprehensive 3D GC-TOFMS system (GC×GC×GC-TOFMS)	^{1}D: Rtx-5 (30 m×0.25 mm I.D.×0.50 µm d_f) ^{2}D: Rtx-Wax	[158]

			(3.5 m×0.18 mm I.D.×0.18 µm d_f) ^{3}D: Rtx-200 (1 m×0.10 mm I.D.×0.10 µm d_f)	
Carrot seed essential oil	High-resolution GC separation and preparative isolation of volatile components	Online 3D preparative MDGC system ($_{prep}$GC–$_{prep}$GC–$_{prep}$GC)	^{1}D: Equity-5 (30 m×0.53 mm I.D.×5 µm d_f) ^{2}D: SUPELCOWAX®10 (30 m×0.53 mm I.D.×2 µm d_f) ^{3}D: SLB-IL59 (30 m×0.53 mm I.D.×0.85 µm d_f)	[159]
Fuel oils	Precise, high-resolution characterisation of multicomponent samples	Hybrid (sequential) GC×GC–MDGC system	^{1}D: SolGel-Wax (30 m×0.32 mm I.D.×0.5 µm d_f) ^2D_M: VF5 (5 m×0.15 mm I.D.×0.15 µm d_f) ^3D_L: Rxi-17Sil ms (20 m×0.18 mm I.D.×0.18 µm d_f)	[160]
Vapor analytes	Exploiting the multidimensional separation capability to a greater extent	Computer-controlled smart 3D micro-GC system	^{1}D: Rtx-5 ms (0.8 m) ^{2}D: Rtx-1 (1.0 m) ^{3}D: SUPELCOWAX®10 (3.0 m)	[161]
Chiral oxime	Investigation of on-column reversible molecular interconversion processes	Online enantioselective 4D dynamic GC system (GC_{enant}×GC_{IL}-*D*GC-GC_{np}–accTOFMS)	$^1D_{enant}$: MEGA-DEX DET-Beta (25 m×0.25 mm I.D.×0.25 µm d_f) $^2D_{IL}$: SLB-IL111 (1.8 m×0.1 mm I.D.×0.1 µm d_f) $^3D_{react}$: SUPELCOWAX®10 (15 m×0.25 mm I.D.×0.25 µm d_f) $^4D_{np}$: DB-5 ms (30 m×0.25 mm I.D.×0.25 µm d_f)	[162]

Heavy petroleum fractions	Quantitative characterisation of petrochemical samples	Online integrated SFC–2GC×GC-FID system Online integrated SFC×2GC×GC-FID system	^{1}D: DB1-HT (10 m×0.32 mm I.D.×0.1 µm d_f) ^{2}D: BPX-50 (0.8 m×0.1 mm I.D.×0.1 µm d_f) ^{1}D: DB1-HT (10 m×0.32 mm I.D.×0.1 µm d_f) ^{2}D: BPX-50 (1.45 m×0.1 mm I.D.×0.1 µm d_f)	[164]
Vetiver essential oil	Rapid preparative isolation of high solute amounts	Online LC with preparative MDGC system (LC–$_{prep}$GC–$_{prep}$GC–$_{prep}$GC)	LC column: SUPELCOSIL LC-Si (250 mm×4.6 mm I.D.×5 µm d_p) GC columns: ^{1}D: Equity-5 (30 m×0.53 mm I.D.×5.0 µm d_f) ^{2}D: SLB-IL59 (30 m×0.53 mm I.D.×0.85 µm d_f) ^{3}D: SUPELCOWAX®10 (30 m×0.53 mm I.D.×2.0 µm d_f)	[166]
Coal tar	Separation and characterisation of extremely complex samples	Online LC with GC×GC and triple quadrupole MS system (LC–GC×GC-QqQMS)	LC column: SUPELCOSIL LC-Si (100 mm×3 mm I.D.×5 µm d_p) GC columns: ^{1}D: SLB-5ms (30 m×0.25 mm I.D.×0.25 µm d_f) ^{2}D: SUPELCOWAX®10 (1 m×0.10 mm I.D.×0.10 µm d_f)	[167]

Must and wine	Quantification of 3-alkyl-2-methoxypyrazines in grape must and wine	Online LC with MDGC and quadrupole MS system (LC–MDGC-MS)	LC column: Hypersil Si (120 mm×2 mm I.D.×3 µm d_p) GC columns: ^{1}D: ZB-Wax (20 m×0.25 mm I.D.×0.5 µm d_f) ^{2}D: ZB-35 (15 m×0.25 mm I.D.×0.5 µm d_f)	[168]

1.5 Scope of the thesis

The primary goal of this thesis was to investigate and develop a range of advanced chemical analysis strategies using GC related techniques (GC×GC, 2GC×2GC, and GC–GC×GC) in conjunction with high resolution MS detection for untargeted and/or targeted profiling of secondary metabolites in hop plants (*Humulus lupulus* L.). These proposed high resolution multidimensional GC separation approaches have succinctly demonstrated great potential in the deep characterisation of hop phytoconstituents (monoterpenes, sesquiterpenes, esters, and ketones), meanwhile which are not limited to plant extracts analysis but can also be easily applied for other complex mixtures characterisation. The thesis introduction and literature review is described in **Chapter 1**. This chapter details hop plant phytocomposition (volatile and nonvolatile), analytical gas chromatographic methods, as well as recent developments in advanced system designs of MD or hyphenated approaches for plant volatiles analysis.

The performance of high resolution GC coupled with Q-TOFMS, which is currently still the mainstay analytical method for plant essential oil analyses, was investigated initially for the qualitative analysis of secondary metabolites in hop essential oils, and the outcome is described in **Chapter 2**. This chapter investigates and details the volatile phytochemical diversity of 30 hop samples obtained from experimental hybrid and commercial *H. lupulus* L. plants. Differences among the analysed samples, in the relative proportions of mono- and sesquiterpenoids, appear to be related to either the genetic origin of the plants or the processes of bioaccumulation of the secondary metabolites identified.

The phytochemical composition of hop makes it a vital raw material for the brewing industry. However, achieving reasonable separation of all components present within the samples, to increase the accuracy and reliability of MS identification, is always a major challenge to hop analysts. **Chapter 3** demonstrates the potential of GC×GC coupled with Q-TOFMS, to perform the untargeted metabolic profiling of hop essential oils from eight representative genotypes. Characteristic compounds that discriminate different hop varieties and the classification of three major chemotypes were evaluated and discussed in this chapter. Comprehensive overview and distinguishable chemotypic patterns was readily observed due to the orderly distributions of metabolites in the 2D separation space. The detailed 3D metabolic fingerprints of different hop genotypes were further interpreted through principle component analysis, allowing simple and fast screening of new hybrid hop genotypes from breeding programs.

In an attempt to dramatically improve the separation and identification capability based on a conventional GC×GC instrument, **Chapter 4** focuses on the investigations of the development of an independent flow controlled parallel 2GC×2GC approach. Appropriate flow control at the junction of ^{1}D and ^{2}D columns permits the possibility and simplicity of implementing GC×GC and 2GC×2GC experiments using 250 μm homologous I.D. column combinations. Comparison of analyses with and without independent ^{2}D flow control, and the importance of applying flow control to adjust the separation speed in ^{2}D were outlined. The capability of the proposed approach was demonstrated by the analyses of hop essential oil with two column combinations. Its important features compared to the reported multiplexed GC approaches were

discussed.

The metabolic compositions in natural plant extracts are of such diversity and complexity which not always anticipated by analysts. The peak capacity of a GC×GC separation will tend to be exceeded in some applications. Development of higher order GC designs with expanded resolving power, able to cater for metabolites of widely different abundance, is of continuing interest. A novel sequential 3D GC hyphenated with Q-TOFMS (3D GC-Q-TOFMS) approach for profiling secondary metabolites in complex plant extracts was described in **Chapter 5**. The integrated system incorporates a preliminary ^{1}D separation step, prior to microfluidic heart-cutting of a targeted region(s) to a ^{2}D column for multidimensional separation (GC–GC). For additional separation, effluent from ^{2}D can then be modulated according to a GC×GC process, to produce a sequential GC–GC×GC separation. The analytical performance and applicability of the proposed approach were demonstrated and discussed through the separation and detection of oxygenated sesquiterpene components in hop essential oil and agarwood (*Aquilaria malaccensis*) oleoresin.

Chapter 6 draws an overall conclusions to the work presented and highlights possible opportunities leading to the future of high resolution MDGC and MS methods for both targeted and untargeted analysis of hop secondary metabolites. Several future studies for unravelling the complex flavour and aromatic components of hop plant are also suggested as a continuation of this study.

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CHAPTER 2

Assessment of the phytochemical profiles of novel hop (*Humulus lupulus* L.) cultivars: A potential route to beer crafting

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CHAPTER 3

Chemotyping of new hop (*Humulus lupulus* L.) genotypes using comprehensive two-dimensional gas chromatography with quadrupole accurate mass time-of-flight mass spectrometry

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CHAPTER 4

Parallel comprehensive two-dimensional gas chromatography

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CHAPTER 5

Sequential hybrid three-dimensional gas chromatography with accurate mass spectrometry: A novel tool for high-resolution characterization of multicomponent samples

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CHAPTER 6

Concluding remarks and future perspectives

6.1 Concluding remarks

The knowledge of chemical components in hop-derived products is deemed as crucial for many purposes, such as for interpretation of metabolic processes, monitoring of related product quality and manufacturing processes, in addition to the exploitation of specific compounds for human benefits. However, the diversity and complexity of hop essential oils are so great that achieving complete separation and detection of all components has been an analytical challenge. This thesis describes the development and application of advanced GC approaches (GC×GC, 2GC×2GC, and GC–GC×GC) incorporating multiple separation and detection dimensions for improved chemical analysis, with a primary focus on hop volatiles.

The early stages of the research (**Chapter 2**) were designed to demonstrate the capabilities and limitations of high resolution one-dimensional GC analysis of complex plant essential oils. This investigation showed that by GC employing a 60 m capillary column with Q-TOFMS detection, the minor or major volatile phytochemical variations among 30 essential oils distilled from experimental hybrid and commercial *H. lupulus* L. plants were attained. Meanwhile, the phytoconstituent diversity among the analysed various hop genotypes was critically evaluated and interpreted in terms of their genetic and biogeographical origins, and the related biosynthetic processes. The observed differences, in terms of the relative proportions of mono- and sesquiterpenoids, was found to be related to either the genetic origin of the plants (European or North American hop genotypes) or the bioaccumulation processes (cytoplasmic or plastidial biosynthesis) of the identified secondary metabolites. This study provides valuable information for the continued development of distinctive hop

character in beer. However, the inability of providing a comprehensive profiling of the complex hop essential oils because of the insufficient peak capacity offered by the single-dimension separation was apparent. Moreover, conducting precise quantification and investigating the influence of sample preparation, cultivation factors, and post-harvest processing on the compositional profile need to be further assessed.

Next, the utilisation of high resolution GC×GC-Q-TOFMS approach as a chemotyping tool for metabolic profiling of secondary metabolites in hop essential oils, focusing on a representative range of genotypes, was investigated (**Chapter 3**). A combination of ^{1}D polar wax and ^{2}D non-polar 5% phenyl polysilphenylene-siloxane phases were chosen to effect the separation, providing a broad overview on the constituents of hop sample. Comprehensive overview and distinct differences of metabolic profiles were readily observed based on 2D or 3D plots. A complex array of secondary metabolites was detected, and oxygenated sesquiterpenes were proposed as a key chemical identifier group for different genotypes. The markedly different metabolite profiles were further interpreted through chemometric analysis, which allow the classification of three major chemotypes among analysed genotypes. By considering the improvements in overall spearation of components (*i.e.*, extension of metabolic coverage) achieved over conventional 1D GC, the proposed high resoltuion approach proves to be adequate for deeper characterisation of the pytochemical compositions in hop. It also represents an important advance in the context of improved methodologies for fast screening of new hybrid hop genotypes from breeding programs.

An independent flow controlled four-column multiplexed approach comprises two parallel and independent two-dimensional column ensembles (2GC×2GC) was

developed to provide enhanced qualitative information over conventional GC×GC method (**Chapter 4**). The first part of this study has demonstrated the performance and benefits of applying precise ^{2}D flow control at the junction of ^{1}D and ^{2}D column, with homologous 250 μm internal diameter to overcome the typical problem of flow-mishmatch, in a GC×GC system. This implementation provided correction of the pressure drop across the entire column set, and in turn have an appropriate correction of flow rate in both dimensions. Moreover, the proposed approach also permits independent control of flow conditions in each dimension of the GC×GC system to maximise resolution by simultaneously achieving close to optimum flow rate in both columns, *e.g*., *Efficiency Optimised Flow* and *Speed Optimised Flow* can be readily applied to ^{1}D and ^{2}D, respectively. The effectiveness of integrating flow control with multiplexed 2GC×2GC system to adjust the separation speed in ^{2}D while using 250 μm I.D. ^{2}D columns, was demonstrated in the seocnd part of this study. The proposed 2GC×2GC-flow control approach was successfully applied for the analysis of hop essential oil with two different column combinations. The two entirely independent GC×GC separations produced by this single detector system provide complementary separation and additional identification information due to the different selectivity of the four separation columns, which is highly promising for qualitative analysis. Coupling 2GC×2GC to MS: four independent retention times (or retention indices) with mass spectrometry should provide unequivocal assignment of individual peak identity in complex samples.

In the last section of this thesis, a sequential hybrid 3D GC-Q-TOFMS approach for high resolution characterisation of complex plant extracts was developed (**Chapter 5**).

The integrated system demonstrated the feasibility of incorporating a preliminary $^{1}D_{np}$ separation step, prior to microfluidic heart-cutting of a targeted region(s) into a $^{2}D_{PEG}$ column for GC_{np}–GC_{PEG} separation. For additional separation, effluent from $^{2}D_{PEG}$ can then be modulated according to a GC×GC process, using an ionic liquid phase as $^{3}D_{IL}$ column, to produce a sequential GC_{np}–GC_{PEG}×GC_{IL} separation. Thus unresolved or poorly resolved components, or regions that require further separation, can be precisely selected and rapidly transferred for additional separation on ^{2}D or ^{3}D columns, based on the greater separation realised by these steps. The analytical performance of the proposed system was evaluated through the separation and detection of oxygenated sesquiterpenes in hop essential oil and agarwood oleoresin. Improved resolution and peak capacity was illustrated through the progressive comparison of tentatively identified components for GC_{np}–GC_{PEG} and GC_{np}–GC_{PEG}×GC_{IL} methods. The described methodology should be a valuable adjunct for improved characterisation of complex plant matrixes, particularly in the area of plant metabolomics.

6.2 Future perspectives

Based on this research work, future research prospects can be suggested as outlined below:

Multiple sequential injection comprehensive three-dimensional GC-MS method for high-resolution profiling of multicomponent samples

Future research is urged to fully utilise the developed hybrid 3D GC-MS platform, as discussed in Chapter 5, to further enhance chromatographic resolution and overall

peak capacity. The developed hybrid 3D GC-MS method has been demonstrated as a useful approach to heart-cut, and rapidly transfer any specific region(s) of interest from ^{1}D column into a ^{2}D or ^{3}D column for additional separation. Based on the current hybrid 3D GC set-up, multiple sequential H/Cs can be implemented with very narrow heart-cut windows (*e.g.*, 0.20 min) over a 10 to 20 min retention window from the ^{1}D chromatogram, and then each H/C is subsequently transferred into the ^{2}D and ^{3}D column for GC×GC separation. Comprehensiveness in terms of 3D separation arises as the whole sample is sampled sequentially from the ^{1}D, then to the ^{2}D and ^{3}D columns for addition separation. Each collected chromatogram can be reconstructed and realigned to be displayed as a comprehensive 3D plot with three retention times for each axis. The H/C zone times and their frequency (time interval between each H/C) will be mostly defined by the sample complexity, the length of the ^{2}D and ^{3}D column, and the extent of retention of components on the ^{2}D and ^{3}D column. With injection of authentic standards, or other methodologies described in the last paragraph of this chapter, quantitative analysis can be conducted for each baseline-resolved component.

Accurate structural identification of hop-derived secondary compounds using preparative H/C MDGC-MS combined with nuclear magnetic resonance (NMR) and/or Fourier transform infrared resonance (FTIR) spectroscopy

Although the identification of individual component tend to be carried out via a combination of RI and MS data, there are circumstances where additional supporting information may still be desired for unambiguous identification, particularly when pure reference materials are not available. As shown in Chapter 3, many terpene

compounds detected in hop essential oil have not been assigned appropriate identities because of the absence of a wider coverage of MS databases or the lack of specificity of spectra (*e.g.*, isomeric species or related compounds). However, these terpene compounds are the main chemical group of interest due to its important relevance to the specific and unique beer flavour impression. The use of preparative MDGC for isolation and collection of individual targeted hop secondary compound(s), in association with spectroscopic analysis for accurate structural confirmation, by using NMR and/or FTIR, could provide absolute certainty when MS is unable to offer sufficient selectivity. This approach will have a considerable role for the elucidation of many undiscovered novel hop-derived secondary compounds, particularly terpene-type structures. Combining with synthesis of novel components, precise quantification can be readily obtained by using MDGC and/or GC×GC approaches.

Portable GC-MS in combination with HS-SPME method for direct on-field measurement of hop volatiles

In the field of hop chemistry, the phytochemical profile of volatile secondary compounds is a key factor in defining both quality and value of hop strobiles, measures to determine phytoconstituents of different hop varieties have been of enormous importance to phytologist and hop breeders. The routine method for hop analysis, such as GC-MS and/or GC×GC-MS, needs long chromatographic separation time by using conventional bench-top instrumentation, as shown in Chapter 2 and 3. Therefore, development of a portable GC-MS in combination with HS-SPME method for the measurement of hop volatile compounds in a short analysis time will be an interest of hop chemists. In this instance, capability to rapidly perform qualitative

screening for basic characterisation of hop chemical phenotypes may facilitate fast selection of novel cultivars and/or discriminating intraspecific diversity of *H. lupulus* (particularly for wild hops) prior to follow-up quantitative analyses using bench-top instrument. With proper implementation, this technique can be efficiently used in the hop farms to allow fast on-site analysis of hops, such as rapidly classifying hop varieties based on their volatile and/or semi-volatile profiles, and monitoring of accumulation of specific secondary compounds during hop ripening progresses.

Rapid quantification of volatile hop-derived flavour compounds using predicted relative-response factors (RRF) combined with multidimensional GC

Nowadays, there is an ever-increasing demand for accurate quantitative analysis in the flavor and fragrance field, particularly essential oils. Having pure authentic standards is the best-case scenario, but which is a quite unrealistic expectation that individual pure standard is available for every compound. The raw percentages of peak areas are often used as such, or in association with that of an internal standard (ISTD), which is a practical approach but has been shown to having limited value. RRF is a widely accepted method by the flavour and fragrance industry for rigorous quantification, but it relies on having a good RRF database as well as applying identical instrumental settings. Recently, the International Organisation of the Flavour Industry (IOFI) recommended the use of predicted RRF as a rapid and reliable method for quantifying constituents in complex mixtures by means of internal standardisation (ISTD: methyl octanoate), without the need of authentic compounds, which further avoid time-consuming calibration procedures. This methodology takes into account of the molecular formula of each compound, but also specific molecular features such as

benzene rings for the prediction of RRF. By combining with multidimensional GC (GC×GC and/or MDGC)-MS/FID, this technique should be a promising approach for hop quantification, enabling precise and rapid quantitative analysis of all the identified hop-derived compounds without the need of pure reference substance.